NOTES

Biofilm Formation by *Neisseria meningitidis*

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Biofilm formation by the human pathogen *Neisseria meningitidis* was analyzed. Biofilm-forming meningococcal strains were identified and quantitated by crystal violet staining. Laser scanning confocal microscopy of the meningococcal biofilm revealed variable layers up to 90 μm in thickness. A total of 39 meningococcal isolates were studied; 23 were nasopharyngeal-carriage isolates, and 16 were invasive-disease isolates. Thirty percent of carriage isolates and 12.5% of invasive-disease isolates formed biofilms proficiently on a polystyrene surface. Generally, the strains that formed biofilms showed high-level cell surface hydrophobicity, characteristic of strains lacking a capsule. The inhibitory role of capsule in biofilm formation was further confirmed by comparing the biofilm-forming capabilities of a serogroup B wild-type strain of a disease-associated isolate to those of its capsule-deficient mutant (*ctrA*). Some strains of meningococci form biofilms, and this process is likely important in meningococcal colonization.

Bacterial biofilms are sessile bacterial communities that adhere to each other and solid surfaces and are enclosed in an exopolysaccharide matrix (6). Biofilms are the predominant communities of many bacterial species in numerous ecosystems. Formation of biofilms involves participation of the extracellular-matrix and cellular-surface molecules, including membrane proteins. Biofilm formation also requires considerable bacterial energy and resources. The formation of biofilms begins with the attachment of the planktonic cells to a suitable surface, followed by replication and spreading. Eventually, the biofilms mature to differentiated forms. Exopolysaccharides play a key role in the establishment of biofilm architecture (6).

In clinical settings, bacteria in biofilms are less susceptible to antimicrobial agents and host immune responses, thereby becoming persistent colonizers or sources of chronic infections (8). Bacteria are released from biofilms as individual planktonic cells or as a result of the sloughing of the biofilms. While many biofilms form on abiotic surfaces such as medical devices, some also develop on living tissues, as in the case of endocarditis or cystic fibrosis (8).

Studies of biofilm formation by the *Neisseria* species are very limited, and most of those species examined have been oral commensals (4, 20, 24, 26, 38). Biofilm formation by *Neisseria meningitidis*, an etiologic agent of epidemic sepsis and bacterial meningitis, has not been documented. Meningococci are isolated from 5 to 10% of the normal population, and the colonization of the human nasopharyngeal mucosal surface by meningococci is the first step of the host-parasite interaction.

Successful meningococcal colonization requires initial attachment facilitated by pili and subsequent interaction of other secondary-surface molecules with the host mucosal surface (12, 31, 36, 43).

In this study, the formation of the biofilms by *N. meningitidis* was assessed. In addition, the roles of the bacterial-surface molecules (pilus, capsule, and lipooligosaccharide [LOS]) in the biofilm formation were also determined. The meningococcal strains used in this study are listed in Table 1. Meningococci were grown on GC medium base (GCB) (Difco) agar containing Kellogg's supplements and incubated at 37°C and 5% CO₂ tension or in liquid cultures in GC broth (1.5% proteose peptone no. 3 [Difco], 0.4% K₂HPO₄ [Sigma], 0.1% KH₂PO₄ [Sigma], and 0.5% NaCl [Sigma] plus Kellogg's supplements I and II) at 37°C and 5% CO₂ with agitation. When necessary, streptomycin (750 $\mu\text{g}/\text{ml}$) was added to the medium.

One of the biofilm-forming strains was selected for the microscopic examination by confocal laser scanning microscopy. A 1:100 dilution of an overnight culture in GCB of strain IR3501 (Table 2), a serogroup B meningococcal-carriage isolate, was inoculated into 10 ml of fresh GCB in a sterile polystyrene 100- by 15-mm petri dish containing sterile borosilicate glass coverslips. The coverslips were removed after overnight incubation at 37°C in a 5% CO₂ atmosphere without agitation and carefully rinsed with fresh GCB. The coverslips were then stained with 30 μg of acridine orange (Sigma)/ml, rinsed again with GCB, and mounted onto a microscope slide as follows: a circle 10 mm in diameter was cut out of the middle of a 25-mm² piece of parafilm, greased on both sides with stopcock grease, and laid on the slide. Fresh GCB was applied to the center well in the parafilm, and the coverslip was fitted over the top of the parafilm gasket. The slide was then observed microscopically. All confocal microscopy experiments were performed on an

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TABLE 1. Genetically modified meningococcal strains used in the study

Strain	Description	Source and/or reference(s)
IR4127	<i>N. meningitidis</i> serogroup B, strain NMB; a derivative of IR2781, spontaneously streptomycin resistant	Yi et al. (46)
IR5389	<i>rfaC</i> ::Km derivative of IR4127	Stojiljkovic et al. (39)
IR5390	<i>ctrA</i> ::Sp derivative of IR4127	Swartley et al. (40)
IR5391	<i>pilQ</i> ::Cm of IR4127	Yi et al. (46)
IR5545	<i>pilQ</i> ::Cm of IR3501	This study

Axiovert 135 inverted microscope equipped with an LSM-410 inverted confocal laser scanning microscope (Carl Zeiss, Jena, Germany). A $\times 40$ 1.2-numerical-aperture C-Apochromat objective lens was used for observing the biofilms. All images were captured with a charge-coupled-device camera. Cross-sections were captured in 2- μ m sections through the 100- μ m sample depth of the *N. meningitidis* biofilm unless otherwise indicated. Excitation and emission were 488 and 510 to 525 nm, respectively. The examination showed a thick layer of meningococci and matrix material that ranged from less than 30 μ m to above 60 μ m (Fig. 1A). A sagittal view of the biofilm additionally shows the thick but variable layer of meningococci and matrix (Fig. 1B). Potential pillars of the biofilms are indicated.

Formation of biofilms by strain IR3501 over a 12-h time course was assessed by Congo red staining and light microscopy (Fig. 2). Congo red stains starch, amylose, and polysaccharides containing contiguous β -(1 $\rightarrow$ 4)-linked D-glucopyranosyl units or β -(1 $\rightarrow$ 3)-D-glucans and has been used to detect exopolysaccharides constituting the extracellular materials of biofilms (28, 41, 45). At various time points, bacterial exopolysaccharides were visualized according to a modification of

the staining method of Harrison-Balestra et al. (18). Ten microliters of an overnight culture was inoculated into 10 ml of fresh GCB in a sterile polystyrene 100- by 15-mm petri dish (1:1,000 dilution) containing sterile borosilicate glass coverslips. At various times, the coverslips were removed, and cetylpyridinium chloride (Sigma) (10 mM) was applied for 30 s and then discarded. The coverslips were air dried for 20 to 30 min. The specimens were stained with a 2:1 (vol/vol) mixture of saturated Congo red (Sigma) solution and 10% Tween 80 (Sigma) for 15 min after gentle heat fixation. Subsequently, the meningococci were stained with 10% Carbol Fuchsin for 6 min. After air drying, the coverslips were mounted on slides and observed by light microscopy as noted above. After 5 h of inoculation, microcolonies were observed (Fig. 2a). The microcolonies expanded to form structures of biofilms and exopolysaccharides after 7 and 9 h of incubation (Fig. 2b and c). This coincides with previous observations that exopolysaccharides are not visible after 5 h or later (10, 17, 44). The biofilms organized after 12 h, and exopolysaccharides were more visible than at earlier time points (Fig. 2d).

In contrast to the results seen with strain IR3501, serogroup B meningococcal strain IR4127 did not form a biofilm (Table

TABLE 2. Meningococcal nasopharyngeal-carriage and invasive-disease isolates

Meningococcal-carriage strain (serogroup) ^a	Biofilm formation assay result (OD ₆₃₀ of CV) ^b	Meningococcal invasive-disease strain (serogroup)	Biofilm formation assay result (OD ₆₃₀ of CV) ^b	Source and/or reference(s)
IR3500 (B).....	+ (0.301)	IR2849 (A)	—	M. Reeves; 35
IR3501 (B).....	+ (0.583)	IR2851 (A)	—	M. Reeves; 35
IR3504 (Y).....	+ (0.116)	IR2854 (Y)	—	M. Reeves; 35
IR3540 (B).....	+ (0.670)	IR2855 (A)	—	M. Reeves; 35
IR5227 (Y).....	+ (0.105)	IR2857 (C)	—	M. Reeves; 35
IR3543 (B).....	+ (0.231)	IR2858 (C)	—	M. Reeves; 35
IR5237 (Y).....	+ (0.109)	IR2859 (C)	+ (0.453)	M. Reeves; 35
IR5235 (Y).....	—	IR2860 (C)	—	M. Reeves; 35
IR5234 (Y).....	—	IR2863 (C)	—	M. Reeves; 35
IR3505 (Y).....	—	IR3474 (A)	—	D. Stephens
IR3542 (B).....	—	IR5450 (Y)	—	D. Stephens
IR3544 (B).....	—	IR5451 (Y)	—	D. Stephens
IR3545 (B).....	—	IR5452 (Y)	—	D. Stephens
IR3546 (B).....	—	IR5453 (Y)	+ (0.097)	D. Stephens
IR3547 (B).....	—	IR5454 (Y)	—	D. Stephens
IR3548 (B).....	—	IR4127 (B)	—	35, 46
IR3549 (B).....	—			
IR5228 (Y).....	—			
IR5230 (Y).....	—			
IR5231 (Y).....	—			
IR5232 (Y).....	—			
IR5233 (Y).....	—			
IR5229 (Y).....	—			

^a All carriage strains are from the Georgia carriage study (22).

^b The values obtained for the negative strains are less than 0.090.

FIG. 1. Confocal laser scanning microscopy of biofilms formed by meningococcal strain IR3501, a nasopharyngeal-carriage isolate. (A) Topographical illustration of the biofilms formed by IR3501. The composite images were computer generated after the biofilms on a coverslip were scanned horizontally by a confocal laser scanning microscope. Virtual color changes represent the height of the biofilm above the coverslip. A color-coded scale of biofilm depth is located in the upper left corner. (B) Sagittal view of a section of the biofilm. Various sizes of potential biofilm pillars are indicated by the letter P.

1 and Fig. 3). To examine the effect of surface-exposed molecules on the formation of biofilms, isogenic mutants of IR4127 were examined. These included mutants that had defects in capsule (*ctrA* mutant; strain IR5390) or that produced truncated LOS (*rfaC* mutant; strain IR5389) (Table 1). To visualize biofilms, bacteria were inoculated at a 1:100 dilution from the overnight culture and grown as described previously for 24 h in 24-well polystyrene plates containing 500 μ l of GCB. The wells containing bacterial culture were stained with 300 μ l of 0.3% crystal violet (CV; Difco) per well for 2 min after two washes with distilled water. The stained wells were subsequently washed with distilled water twice to remove residual CV. The stained biofilms were dissolved in 33% acetic acid and quantitated by measuring optical density at 630 nm (OD_{630}) (19). The proficiency of biofilms was quantitated by measuring the OD of dissolved CV in acetic acid. The OD values from the wells that had not been inoculated with bacteria were used as the negative control. The cutoff value for determining a biofilm producer was set as two times the negative-control value. The capsule-deficient mutant formed biofilms which were visual-

ized by CV staining (Fig. 3B). The *rfaC* mutant with a KDO₂-lipid A LOS molecule also formed a biofilm at levels lower than those seen with the capsule mutant strain (Fig. 3A). These results indicated that the capsule and LOS oligosaccharide α - and β -chain structures play inhibitory roles in the biofilm formation and that meningococci can develop biofilm most effectively when capsule is absent or LOS truncated. The absence of the negatively charged capsule, in particular, suggests that hydrophobic interactions may play a significant role in biofilm formation.

To better understand the role of hydrophobicity in meningococcal biofilm formation, cell surface hydrophobicity was measured for selected strains. The surface hydrophobicity of meningococcal strains was measured using a modification of a previous protocol (21). Disposable plastic columns packed with octyl Sepharose CL-4B (Sigma) to a height of 2 cm were washed with 10 ml of buffer A (0.2 M ammonium sulfate in 10 mM sodium phosphate buffer; pH 6.8). Meningococci collected from overnight plate cultures were suspended in phosphate-buffered saline to an OD of 10, and a 100-ml aliquot was

FIG. 2. Microscopic views of the meningococcal biofilm formation of strain 3501 ($\times 1,000$ magnification, light microscopy). The process of biofilm formation was visualized by double staining meningococci grown on coverslips with Congo red and Carbol Fuchsin at 5 h (a), 7 h (b), 9 h (c), and 12 h (d). Congo red stains the exopolysaccharides pink (indicated by arrows). Meningococci were stained purple.

gently pipetted onto the surface of the column and eluted with 5 ml of buffer A. A 100- μ l cell suspension diluted directly into 5 ml of buffer A was also prepared as a control. The OD_{600} of both the column flowthrough and control samples was determined. Results were calculated as the OD_{600} of the flowthrough divided by that of the control and expressed as the percentage of meningococci adsorbed to the column. The amount of biofilm determined by CV staining was compared with the cell surface hydrophobicity (Fig. 4). The plot indicates that the proficiency of biofilm formation correlates with the surface hydrophobicity.

A *pilQ* mutant (IR5545) of the biofilm-forming strain (IR3501) was examined to evaluate the role of pili in biofilm formation. PilQ is part of the pilus secretion system (16) and is required for pilus formation. The *pilQ* mutant was nonpilated

and developed biofilms on the polystyrene surface that were indistinguishable in the way that they stained with CV from the biofilms of the parent strain. However, further analysis of the *pilQ* mutant biofilm by confocal laser-scanning microscopy revealed that the mutant formed biofilms that ranged from 20 to 30 μ m in thickness (data not shown). This indicates that the meningococcal pilus may affect the architecture of the biofilm but not the quantity of the biofilm.

A total of 16 disease strains and 23 carriage strains were screened for biofilm formation on the polystyrene surface by crystal violet staining (Table 2). Of these, 30% (7 of 23) of the carriage strains formed biofilms whereas 12% (2 of 16) of invasive-disease isolates formed biofilms, suggesting that biofilm-forming strains are found more often in carriage strains.

FIG. 3. Crystal violet staining of biofilms and quantitation. Biofilms formed by meningococcal mutants of *rfaC* (strain IR5389) and *ctrA* (strain IR5390) were compared with those formed by the parent strain (IR4127 [strain NMB, an invasive-disease isolate]) by visualization with crystal violet staining on a polystyrene surface (B). The amount of biofilm was quantitatively measured as readings at OD₆₃₀ (x axis) (A).

Conclusions. Bacterial biofilms are found on a range of biotic and abiotic surfaces (2, 8, 23). Biofilms can consist of a single species or multiple species that show commensalism and competitive behavior within the biofilm milieu (5). While mixed-species biofilms predominate in nature, single-species biofilms can be found in clinical settings, including medical implants (1, 3, 13). The present study focused on a single-species biofilm formed by the human pathogen *N. meningitidis*. Typical architectural structures of the biofilms reveal water channels between pillars, which are used to deliver the nutrients necessary for bacterial survival (7). Meningococci formed pillars of cells that are approximately 50 to 60 μm in height. Cell surface molecules, including pili, flagella, lipopolysaccharides (LPS), and outer membrane proteins as well as secreted materials such as exopolysaccharides, are involved in the formation of the biofilms in *Pseudomonas aeruginosa* and *Escherichia coli* (9, 14, 25, 27, 32, 33). The identity of the meningococcal exopolysaccharides is of interest. No homologous genes

were identified in a search of the meningococcal genome for the genes encoding colanic acid of *E. coli* and alginate of *P. aeruginosa*.

The role of pili in the formation of biofilms in *P. aeruginosa* and *E. coli* has been extensively studied (14, 33, 34). Type IV pilus mutants of *P. aeruginosa* do not form biofilms, suggesting that pili are required for biofilm formation (33). However, growth on citrate minimal medium eliminates the need for pili on initial attachment or microcolony formation on surfaces and pilus-deficient mutants can form flat biofilms (25). This study also showed that twitching motility mediated by type IV pili was responsible for the migration of the microcolonies. The meningococcal *pilQ* mutant used in this study formed biofilms with a structure thinner than that of the biofilms formed by the wild-type strain. Altered biofilm structure formed by the *pilQ* mutant indicates a potential role for pili in meningococcal biofilm formation.

LPS also seems to play an inhibitory role in the biofilm

FIG. 4. Correlation between cell surface hydrophobicity and biofilm formation. Four biofilm-negative strains and eight biofilm-forming strains were assessed for their percent cell surface hydrophobicity (CSH) (y axis) versus biofilm formation as determined by crystal violet quantification in OD₆₃₀ units (x axis). CSH is presented as the percentage of retained bacteria in the octyl-Sepharose column. The trend is shown as a straight line, with correlation coefficient r^2 equal to 0.8056.

formation. Mutants of *Salmonella enterica* that produce truncated LPS structures form more proficient biofilms than the wild-type bacteria that produce elongated LPS structures (29). This agrees with our observation that the meningococcal LOS mutant (*rfaC*) is more proficient in biofilm formation than its parental strain. The mutant has a truncated LOS structure (KDO₂-lipid A) devoid of heptose and α - and β -chain oligosaccharides, including the α -chain-terminal sialic acid. The absence of the structures may attenuate the steric hindrance and negative charges on the meningococcal surfaces, thereby allowing increased intimate contact between bacterial cells. This hypothesis was further supported by our observation that the LOS mutant autoaggregates more rapidly than the parental strain.

Our study indicates that prevalence of biofilm formation among carriage isolates is greater than that of disease isolates. Not surprisingly, this may be due to capsular expression. The serotyping and surface hydrophobicity profiles suggest that the majority of the non-biofilm-forming strains are encapsulated. Further, in contrast to the encapsulated parent a mutant defective in capsule expression gained the ability to form a biofilm. This suggests that all meningococci have the necessary cellular machinery to form biofilms, for example, when phase variation mechanisms (e.g., slipped strand mispairing) turn off capsule expression. The maintenance of biofilm-forming capabilities despite a metabolic burden suggests a selective advantage. Several lines of evidence suggest that the absence or down regulation of capsule promotes meningococcal colonization, possibly aided by biofilm formation. In natural settings,

approximately 30% of the carriage strains are nonserogroupable (15). Following a community-based intervention program, a higher percentage of *ctrA*-negative isolates was recovered from a school population under study, suggesting that unencapsulated strains recolonize more rapidly than capsulated strains (37). At the molecular level, genes for capsule biosynthesis are down regulated upon contact with the host epithelium, facilitating meningococcal adherence (11). Additionally, gene transfer occurs more efficiently in the biofilms (30); this could also be a selective advantage for meningococci in the nasopharynx.

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